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Effects of microRNA-30a on migration, invasion and prognosis of hepatocellular carcinoma

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ABSTRACT

The role of microRNA-30a (miR-30a) deregulation in tumor progression and its downstream signaling pathways remain unknown. Here we confirmed significant downregulation of miR-30a in hepatocellular carcinoma (HCC) tissues and cell lines compared with non-tumor counterparts. MiR-30a downregulation was significantly associated with worse disease-free survival (DFS) of HCC patients. Gain- and loss-of-function studies revealed that downregulation of miR-30a facilitated tumor cell migration, invasion and epithelial–mesenchymal transition (EMT). We identified SNAI1 as a direct target of miR-30a and demonstrated miR-30a as a novel regulator of EMT by targeting SNAI1, indicating its potential therapeutic value for reducing invasion and metastasis of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is currently one of the most common malignancies and the leading cause of cancer death worldwide [1–3]. Multiple studies have confirmed the aberrant expression of small noncoding microRNAs (miRNAs) in HCC [4–8]. These findings suggest that downregulated miRNAs may be associated with tumorigenesis and progression in HCC. Recent reports demonstrated that the expression of miR-30 family members (miR-30a/b/c/d/e) was significantly reduced in several human cancers, and this reduction also indicated a poor prognosis [9–11]. We previously reported that miR-30 family members, especially miR-30a, were significantly downregulated in HCC tissues. However, the role of miR-30a deregulation in HCC and the molecular mechanisms by which miR-30a exerts its function are still unknown.

Invasion and metastasis are major causes of death in patients suffering from HCC. The process of transition from an epithelial to a mesenchymal state, known as the epithelial–mesenchymal transition (EMT), is a major characteristic of metastasizing cells [12]. During EMT, the loss of epithelial functions and the acquisition of mesenchymal functions result in increased cell migration and invasion. E-cadherin and vimentin function as representative epithelial-specific and mesenchymal-specific protein markers, respectively. Reduced E-cadherin protein expression and increased expression of vimentin protein are used as markers indicating an epithelial cell has undergone EMT [13]. Recent studies suggest that EMT associated with poor prognosis in cancer patients is a key step in the invasion and metastasis of tumor cells [10,14,15].

SNAI1, one of the EMT-associated transcription factors, inhibits E-cadherin expression and promotes tumor cell migration and invasion [16]. Aberrant expression of SNAI1 during EMT was detected in several cancer cell lines. In addition, SNAI1-induced E-cadherin downregulation has been demonstrated essential for triggering EMT [17]. In recent years, increasing evidence has shown that SNAI1 plays an essential role in regulating EMT.

In this study, we showed that miR-30a expression was reduced in the majority of examined HCC tissues, and its downregulation was significantly associated with worse DFS of HCC patients. Furthermore, both gain- and loss-of-function studies revealed that downregulation of miR-30a could facilitate tumor migration and invasion by regulating EMT. Moreover, we found that the SNAI1 transcription factor was a direct target of miR-30a, and it was involved in the effect of reduced miR-30a in enhancing cell migration, invasion and EMT changes. Our findings will help to elucidate the functions of miR-30a and its roles in malignant tumor progression.

Abbreviations: HCC, hepatocellular carcinoma; EMT, epithelial–mesenchymal transition; miRNA, microRNA; 3′-UTR, 3′ untranslated region; qPCR, real-time quantitative polymerase chain reaction; DFS, disease-free survival; TNM, tumor-node-metastasis

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2. Materials and methods

2.1. Tissue specimens

Human HCC tissues and para-cancerous tissues were collected from 63 patients undergoing resection of HCC at the Center of Hepatobiliary Surgery of the First Affiliated Hospital of Xi'an Jiaotong University, P.R. China. The patient characteristics are shown in Supplementary Table 1. Informed consent was obtained from each patient and the study was approved by the Xi'an Jiaotong University Ethics Committee. Detailed information is provided in the Supplementary Materials and Methods.

2.2. Cell lines

MHCC97H, Hep3B, SMMC7721, HepG2, Huh7 (all HCC cells), LO2 (normal human liver cells), and HEK293T (transformed human embryonic kidney cells) were obtained from Fudan University of China and maintained in our lab. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT).

2.3. Transfection of miRNA mimics and inhibitors

Cells were seeded at 1×10^5 cells per well in a six-well plate and transfected with synthetic miRNA mimics or inhibitors (Biomics, Nantong, China) at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA and protein were collected 3 days post-transfection for experimental analyses. The sequences of miR-30a were as follows: sense 5′-GAAGGUCAGCUCCUACAAAUGU-3′ and antisense 5′-AACAUUUGUAGGG CUGACCUUC-3′; the sequence of the miR-30a inhibitor was as follows: 5′-AACAUUUGUAGGAGCUGACCUUC-3′. The sequences of miR-NC were as follows: sense 5′-UUCUCCGAACGUGUCACGUdTdT-3′ and antisense 5′-ACGUGACACGUUCGGAGAAdTdT-3′; the sequence of the anti-miR-NC was as follows: 5′-GUGGAUAUUGUUGCCAUCA-3′.

2.4. Wound healing assay

HCC cells were seeded onto 6-well plates and cultured to confluency. Scratch wounds were made with a 1000-μl pipette tip. The wounds were photographed with a phase-contrast microscope

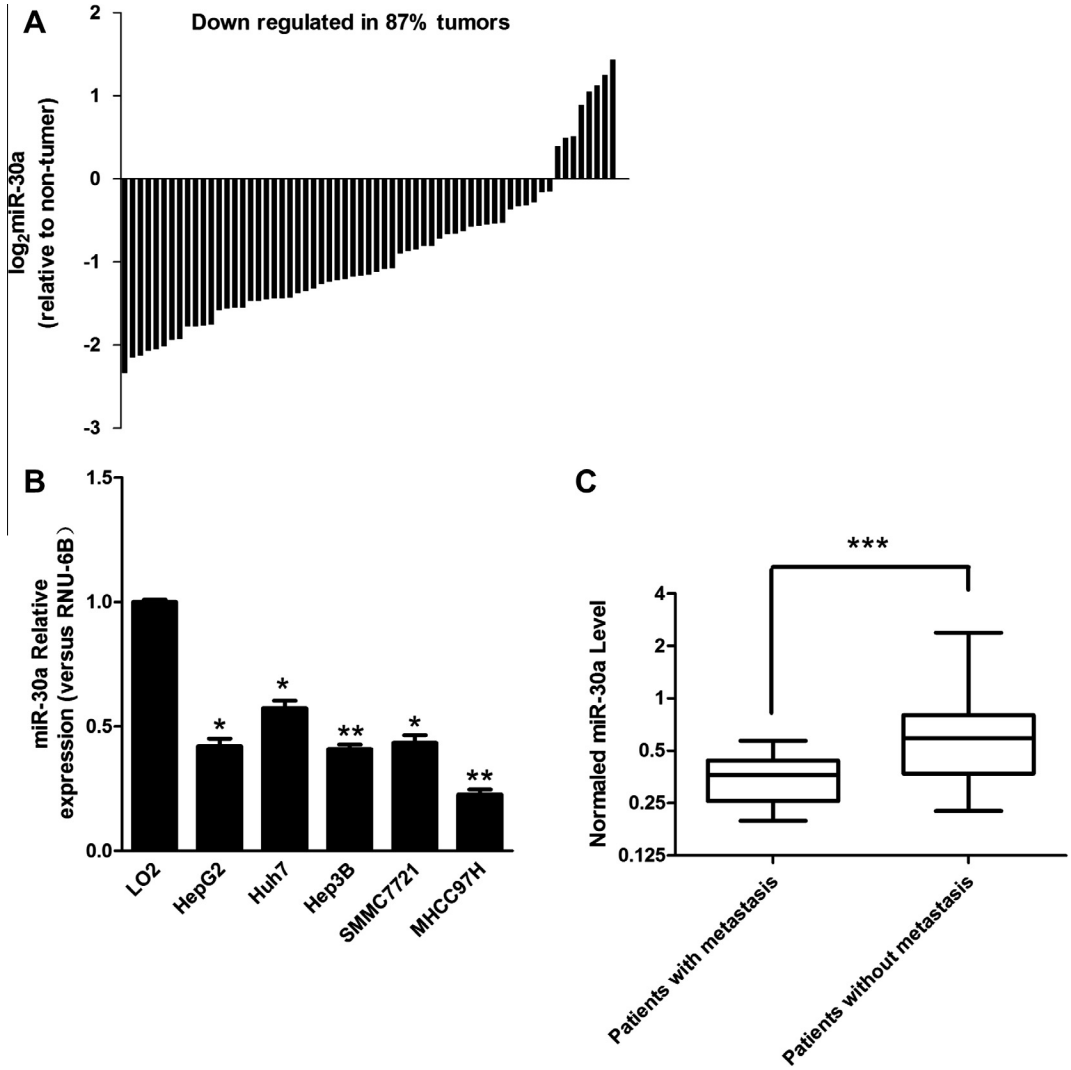

Fig. 1. Expression analysis of miR-30a in HCC tissues and cell lines. (A) MiR-30a was measured by qPCR in total RNA isolated from 63 pairs of HCC tissues and para-cancerous tissues. Relative expression was determined (delta-delta Ct) compared with the internal control RNU6B, and results are presented as fold change ($\log_2$) compared with the expression in the corresponding para-cancerous tissues. MiR-30a expression was downregulated in 87% (55/63) of the HCC cases examined. (B) MiR-30a was downregulated in HCC cells compared with LO2 normal human liver cells. Total RNA from LO2, HepG2, Huh7, Hep3B, SMMC7721 and MHCC97H cell lines were analyzed for miR-30a expression by qPCR. Relative expression was determined (delta-delta Ct) compared with the internal control RNU6B. Data are presented as mean ± SEM ($n = 3$). (C) The levels of miR-30a in HCC tissues with metastasis ($n = 18$) were significantly lower than in those without metastasis ($n = 45$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

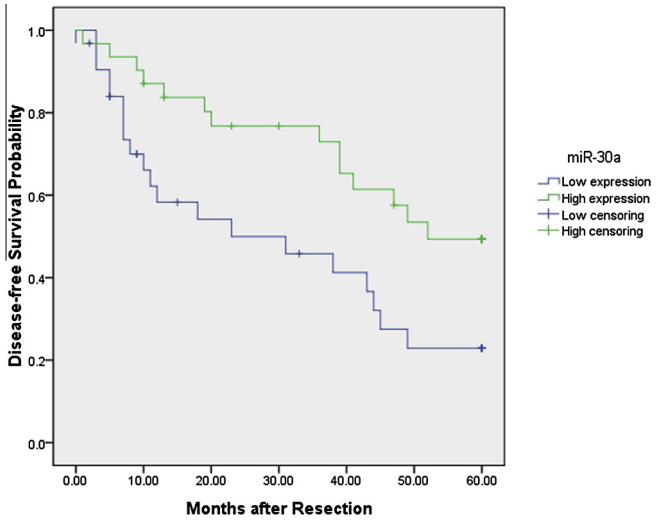

Fig. 2. Kaplan–Meier DFS analysis for HCC patients according to miR-30a level. The miR-30a level was analyzed by qPCR and the median value of all 63 cases was chosen as the cut-off point for separating miR-30a low-expression tumors ($n = 32$) from miR-30a high-expression cases ($n = 31$). $P = 0.015$.

Table 1
Univariate and multivariate analysis of factors associated with disease free survival.[a]

Clinical variables	Case number	HR (95% CI)	*P* value[c]
Univariate analysis			
MiR-30a (low versus high)[b]	32/31	2.3(1.1–4.6)	**0.019**
Age (⩾50 versus <50 years)	27/36	1.0(0.5–2.0)	0.910
Sex (M versus F)	53/10	1.6(0.6–4.7)	0.353
HBV (positive versus negative)	54/9	1.0(0.4–2.7)	0.904
Cirrhosis (yes versus no)	57/6	1.2(0.4–3.8)	0.810
Ascites (yes versus no)	12/51	1.3(0.6–2.9)	0.481
Portal vein tumor thrombus (yes versus no)	8/55	3.3(1.3–8.4)	**0.012**
AFP (⩾400 versus <400 ng/mL)	34/29	1.2(0.6–2.3)	0.639
ALT (⩾50 versus <50 U/L)	37/26	1.0(0.5–1.9)	0.891
Intrahepatic metastasis (yes versus no)	44/19	1.6(0.8–3.5)	0.209
Hepatic capsule invasion (yes versus no)	9/54	0.9(0.3–2.5)	0.792
Tumor size (⩾5 cm versus <5 cm)	41/22	2.6(1.2–5.7)	**0.020**
Tumor number (>1 versus 1)	13/50	0.7(0.3–1.7)	0.458
TNM stage (II/III versus I)	33/30	2.4(1.2–4.9)	**0.014**
Edmondson grade (>II versus I-II)	37/26	2.3(1.1–4.7)	**0.006**
Multivariate analysis			
MiR-30a (low versus high)[b]	32/31	3.2(1.5–6.8)	**0.002**
Portal vein tumor thrombus (yes versus no)	8/55	2.4(0.9–6.4)	0.082
Tumor size (⩾5 cm versus <5 cm)	41/22	2.9(1.3–6.7)	**0.013**
TNM stage (II/III versus I)	33/30	2.0(1.0–4.3)	0.060
Edmondson grade (>II versus I-II)	37/26	2.8(1.3–6.1)	**0.008**

[a] Analysis was performed on 63 cases. Hazard ratios (95% confidence interval) and *P* values were calculated using Univariate or multivariate Cox proportional hazards regression.
[b] The miR-30a level was examined by real-time qPCR and normalized to U6 level. The median value of all 63 samples was chosen as the cut-off point for separating miR-30a low tumors from miR-30a high cases.
[c] Boldface *P* values indicate statistical significant.

at 0, 24 and 48 h. Cell migration was quantitated by measuring the width of the wounds. Migration was calculated as (%) = [*W (0 h) − *W (24 h or 48 h)]/W (0 h), in which *W (0 h, 24 h or 48 h) indicates the width of the wound at 0 h, 24 h or 48 h, respectively. The experiments were performed with at least five replicates.

2.5. Transwell invasive assay

The invasion assay was performed using 24-well Transwell units (BD Biosciences) with 8 μm porosity polycarbonate filters. All the filters were coated with 50 μg reconstituted basement membrane matrix (Matrigel; BD Biosciences). Details are in the Supplementary Materials and Methods.

2.6. RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA from cultured cells was extracted using Trizol (Life Technologies, CA, USA), and total RNA was extracted from HCC tissues using a High Pure miRNA Isolation Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. qPCR was performed as described in the Supplementary Materials and Methods.

2.7. Immunohistochemical analysis

Paraffin-embedded, formalin-fixed tissues were immunostained for E-cadherin, vimentin and SNAI1 as described in the Supplementary Materials and Methods. Staining was evaluated under a light microscope at a magnification of 400×. For each specimen, five images were quantified using image optical density (IOD) by Image-Pro plus software (version 6.0). Measurements of each sample are the mean IOD of all the five images (mean IOD = IOD SUM/5).

2.8. Luciferase reporter assay

The luciferase assay was performed in HEK293T and MHCC97H cells as described in the Supplementary Materials and Methods.

2.9. Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% TritonX-100, 5 mM ethylenediaminetetraacetic acid). Protein concentration was determined using the BCA Kit (Pierce, IL, USA). Protein samples (30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blots were then probed with antibodies against E-cadherin (1:1000; Cell Signaling Technology, MA, USA), vimentin (1:1000; Cell Signaling Technology), SNAI1 (1:1000; Cell Signaling Technology) and β-actin (1:1500, Boster, Wuhan, China). Blots were then incubated with the appropriate fluorescent secondary antibody (1:7000; Boster). Images were acquired by the Bio-Rad Gel imaging system and analyzed by the software program as specified by Bio-Rad.

2.10. Rescue experiment

Functional rescue experiments were performed by cotransfection of miR-30a or anti-miR-30a with plasmid constructs expressing SNAI1. EMT-related protein markers and migration and invasion of MHCC97H cells were then measured. Details are in the Supplementary Materials and Methods.

2.11. Statistical analysis

All statistical calculations were carried out using SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL). *P* values < 0.05 were considered significant, and all tests were two-sided. Kaplan–Meier curves were used to estimate DFS.

3. Results

3.1. Downregulation of miR-30a is a frequent event in HCC tissues and associated with worse prognosis

In a preliminary experiment, we observed that miR-30 family members (miR-30a/b/c/d/e), especially miR-30a, were significantly

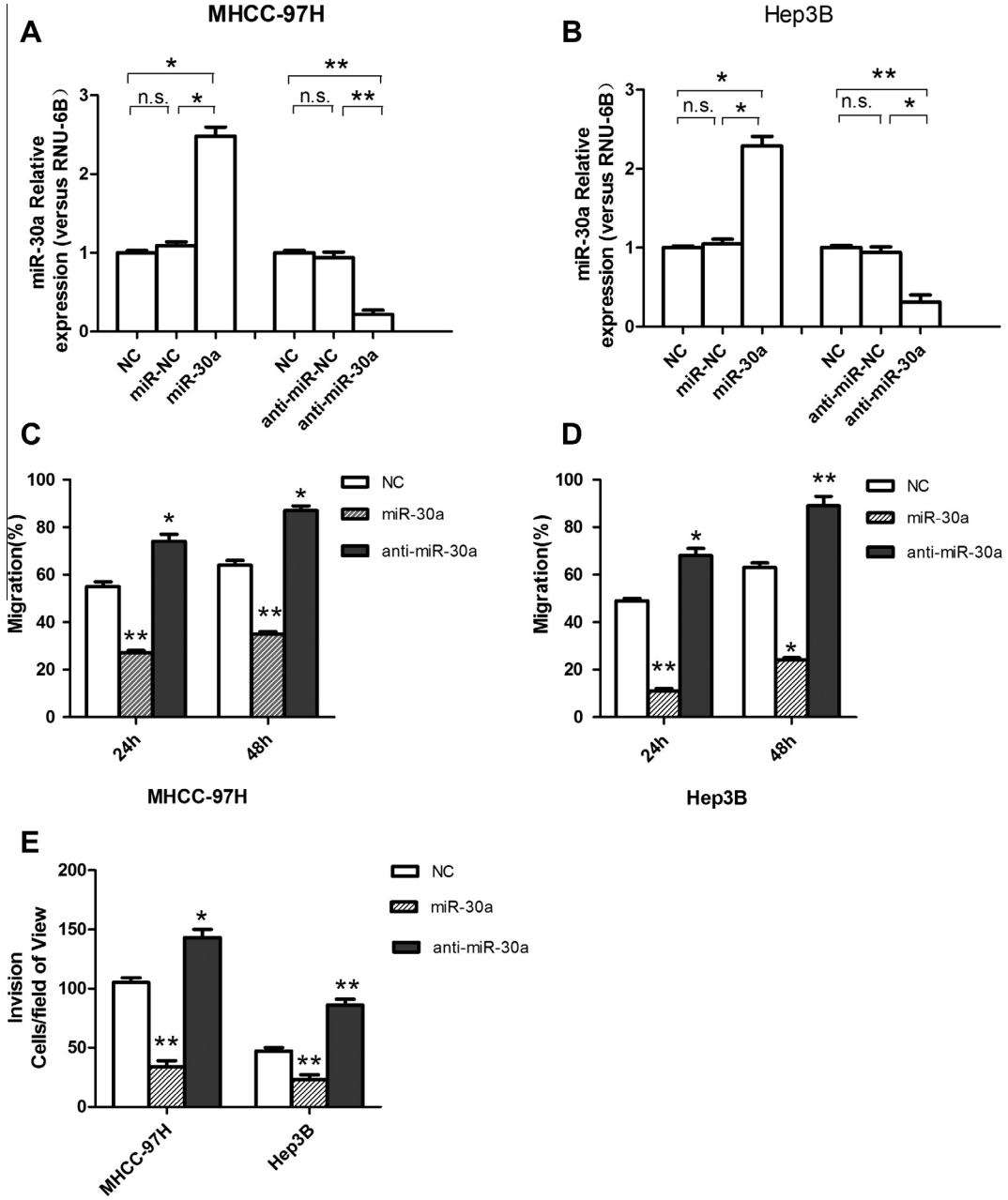

Fig. 3. MiR-30a inhibits migration, invasion and metastasis of MHCC97H and Hep3B cells. After transfection of MHCC97H and Hep3B cells with (A) miR-30a mimics or (B) inhibitors for 3 days, extracted total RNA was analyzed by qPCR to confirm augmented or attenuated miR-30a expression, respectively. Data are presented as relative expression (delta-delta Ct compared with RNU6B). (C and D) The migration capacity of MHCC97H and Hep3B cells was assessed by the wound healing assay. (E) Invasive ability of MHCC97H and Hep3B cells was evaluated by transwell invasive assay. In (C–E), cells without transfection were used as negative controls. Data are presented as mean ± SEM ($n = 3$). n.s. indicates not significant. $^{*}P < 0.05$, $^{**}P < 0.01$.

downregulated in HCC compared with para-cancerous tissues (data not shown). Here we further examined miR-30a expression in 63 paired HCC and para-cancerous tissues using qPCR. Notably, miR-30a was downregulated in 87% (55/63) of the examined HCC tissues (Fig. 1A). Next we analyzed the levels of miR-30a in several HCC cell lines, including HepG2, Huh7, Hep3B, SMMC7721 and MHCC97H, and the normal human liver cell line LO2. Consistent with the above results, qPCR analysis revealed a similar decrease of miR-30a in multiple HCC cell lines compared with LO2 cells (Fig. 1B).

We next investigated whether miR-30a downregulation was correlated with clinical features or prognosis of HCC patients. An association between decreased miR-30a expression and intrahepatic metastasis, advanced tumor-node-metastasis (TNM) stage and high Edmonson pathological classification was observed (Supplementary Table 1). Furthermore, lower miR-30a level was associated with shorter DFS ($P = 0.015$) as determined by the Kaplan–Meier method (Fig. 2). To exclude the confounder effect, we further performed Cox proportional hazards regression analysis. Univariate analysis was first conducted to identify factors that affected DFS, followed by multivariate analysis, which controlled for potential confounders (Table 1). Strikingly, multivariate analysis further confirmed that reduced miR-30a level is an independent predictor for shorter DFS of HCC patients (HR = 3.2; $P = 0.002$) (Table 1).

We next evaluated the levels of miR-30a in HCC tissues with or without metastasis. The levels of miR-30a in HCC tissues of patients with metastasis were approximately 1.1-fold lower than

those of patients without metastasis (Fig. 1C), suggesting that low miR-30a expression level was related to metastasis in patients with HCC. This result indicates that the loss of miR-30a may contribute to metastasis of HCC.

Collectively, these data suggest that deregulation of miR-30a may contribute to the development of HCC.

3.2. Downregulation of miR-30a promotes cell migration, invasion and epithelial–mesenchymal transition (EMT) in HCC

The above findings prompted us to explore the biological significance of miR-30a in tumorigenesis and progression. It is well known that a high frequency of intrahepatic and extrahepatic metastasis in the early stage contributes to poor prognosis of HCC patients, which is also the critical factor involved in the disappointing survival following curative liver resection [18]. Thus, it is necessary to establish the factors that act as HCC metastatic markers. We therefore analyzed the effect of miR-30a on the migration and invasion of HCC cells.

Quantitation of miR-30a expression after transfection with miR-30a mimics confirmed miR-30a overexpression (Fig. 3A) and decreased endogenous miR-30a in HCC cells transfected with miR-30a inhibitors (Fig. 3B). The wound healing assay and transwell invasive assay revealed that increased miR-30a resulted in significantly inhibited migration and invasion in MHCC97H and Hep3B cells (Fig. 3C–E). Furthermore, transfection of MHCC97H and Hep3B cells with anti-miR-30a promoted cell migration and invasion compared with negative controls (Fig. 3C–E). Representative images of migration and invasion are shown in Supplementary Fig. 1A and B and Supplementary Fig. 2. Together these results revealed that downregulation of miR-30a could promote HCC cell migration and invasion.

During tumor progression, cancer cells can acquire motility and invasive ability by initiating the EMT process, which can potentially lead to distant metastases [19]. MiRNAs are emerging as major regulators of tumorigenesis and have also been linked to the regulation of EMT in the development of cancer [20,21]. To investigate whether miR-30a is a crucial regulator of EMT, MHCC-97H and Hep3B cells were transfected with miR-30a mimics or inhibitors. As shown in Fig. 4, overexpression of miR-30a in MHCC-97H and Hep3B cells promoted the expression of E-cadherin and decreased expression of vimentin, which is associated with reversed EMT. Conversely, knockdown of miR-30a in MHCC-97H and Hep3B cells by transfection with miR-30a inhibitors resulted in reduced expression of E-cadherin and increased expression of vimentin, suggesting that reduced miR-30a promotes EMT (Fig. 4).

We further analyzed the associations among miR-30a level, E-cadherin expression and vimentin expression in human HCC tissues. Samples from 63 HCC cases, in which miR-30a levels had been previously determined, were immunohistochemically analyzed for E-cadherin and vimentin expression (Fig. 5A). We confirmed a positive relationship between the expression of miR-30a and E-cadherin in HCC tissues, indicating that miR-30a downregulation was significantly associated with lower E-cadherin levels (Fig. 5B, Supplementary Fig. 3A). Furthermore, miR-30a levels were inversely correlated with vimentin expression (Fig. 5C, Supplementary Fig. 3B).

Taken together, our data confirm that downregulation of miR-30a may be responsible for the progression of EMT in HCC, which in turn promotes migration and invasion of HCC cells.

3.3. MiR-30a directly regulates SNAI1 expression

Next we explored the molecular mechanisms responsible for the functions of miR-30a observed above. Predicted target genes of miR-30a were retrieved and analyzed using publicly available databases (TargetScan and Miranda). The SNAI1 transcription factor, which is known to be relevant to EMT and tumor metastasis, was predicted as one of the targets of miR-30a (Fig. 6A). This prediction was validated using dual-luciferase reporter gene assays in HEK293T cells. Cotransfection of miR-30a significantly suppressed the activity of a luciferase reporter containing the wild-type 3′-UTR of SNAI1 but not that of the mutant reporter (Fig. 6B). In addition, inhibition of endogenous miR-30a by anti-miR-30 led to increased luciferase activity of the wild-type reporter but not the mutant reporter (Fig. 6C). These results were reproducible in MHCC97H cells (Supplementary Fig. 4A and B).

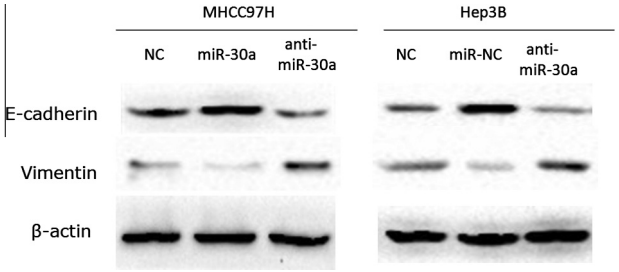

Fig. 4. MiR-30a prevents EMT in MHCC97H and Hep3B cells. Overexpression of miR-30a increased E-cadherin protein expression and downregulated vimentin protein expression significantly in both MHCC97H and Hep3B cells. Silencing miR-30a reduced E-cadherin expression and increased the expression of vimentin in both MHCC97H and Hep3B cells. Non-transfected cells were used as a negative control.

Further investigation showed that cells transfected with miR-30a showed reduced expression of endogenous SNAI1 protein and mRNA (Fig. 6D). In addition, antagonism of endogenous miR-30a resulted in the upregulation of SNAI1 protein and mRNA (Fig. 6D). The correlation between miR-30a levels and SNAI1 levels was further examined in HCC tissues. SNAI1 was analyzed by immunohistochemistry and miR-30a levels were evaluated by qPCR in the same set of specimens shown in Fig. 1A. Notably, miR-30a level was inversely correlated with SNAI1 expression (Fig. 6E and F, Supplementary Fig. 5).

Together, these data suggest that miR-30a may negatively regulate the expression of SNAI1 by directly targeting its 3′-UTR.

3.4. SNAI1 is involved in the effects of reduced miR-30a on promoting cell migration, invasion and EMT changes

To evaluate whether SNAI1 is involved in the effects of reduced miR-30a on increased migration and invasion, MHCC97H cells were transfected with plasmids expressing SNAI1, which resulted in greatly increased SNAI1 protein levels (Fig. 7A). Ectopic expression of SNAI1 abrogated the inhibitory effect of miR-30a on migration and invasion, and further enhanced the effect of anti-miR-30a on promoting migration and invasion in MHCC97H cells (Fig. 7B and C). Representative images of migration and invasion are shown in Supplementary Fig. 6A and B.

We next investigated whether overexpression of SNAI1 could counteract the inhibitory function of miR-30a on EMT. Plasmids encoding SNAI1 were cotransfected with miR-30a or anti-miR-30a into MHCC97H cells, and the expression of SNAI1, E-cadherin and vimentin was analyzed by Western blot. Notably, overexpression of SNAI1 abrogated the effect of miR-30a on changes in protein markers associated with EMT, and strengthened the effect of anti-miR-30a on EMT changes in MHCC97H cells (Fig. 7A). These observations suggest that SNAI1 is potentially involved in the miR-30a-regulated EMT process. Taken together, our data suggest that downregulation of miR-30a may stimulate migration and invasion by promoting the expression of SNAI1, and in turn, facilitating the EMT process.

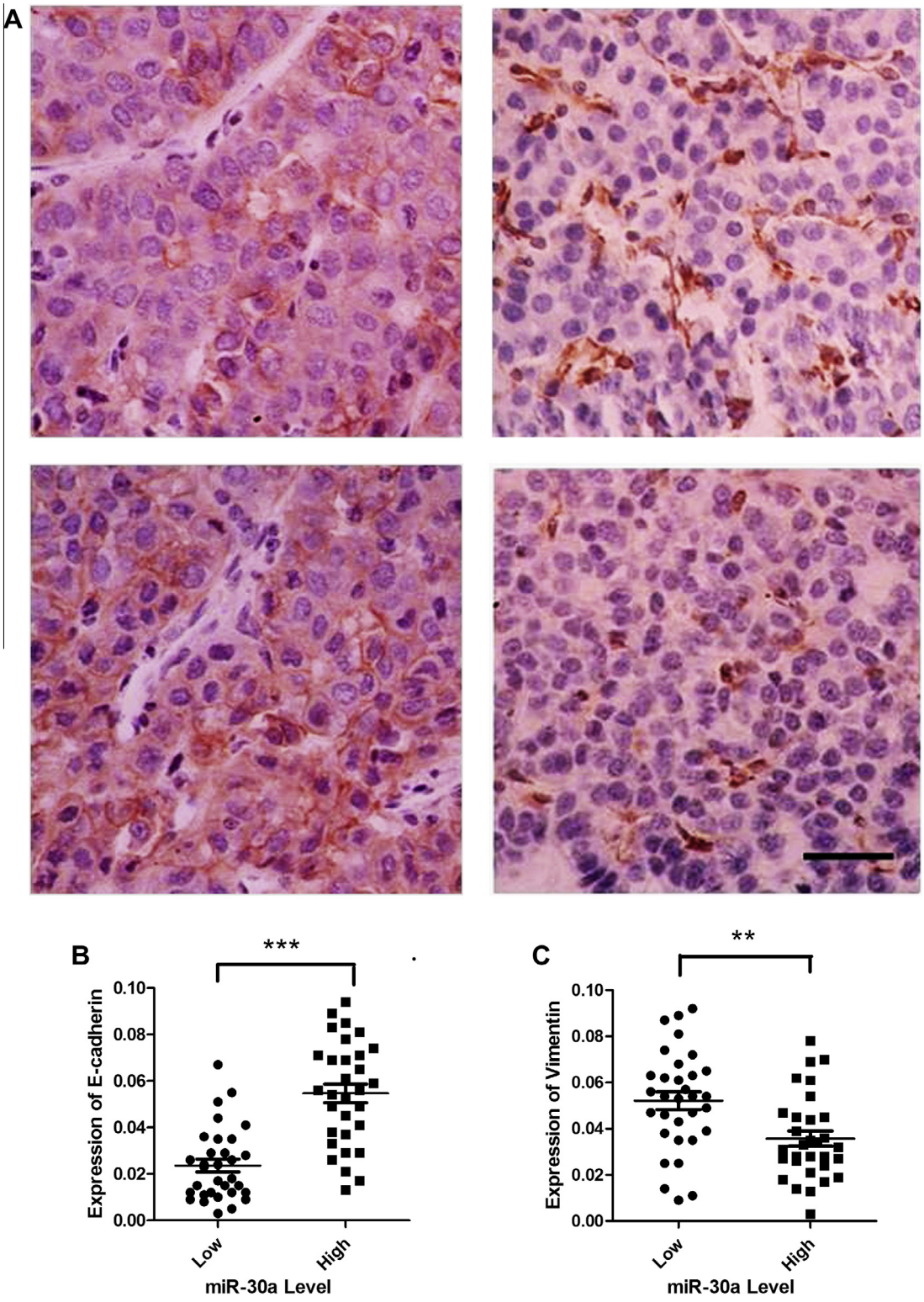

Fig. 5. MiR-30a levels positively correlated with E-cadherin expression and inversely correlated with vimentin expression in human HCC tissues. (A) Representative images of immunohistochemical staining for E-cadherin and vimentin. Magnification, ×400. Scale bar = 50 μm. (B) HCC tumors with lower miR-30a level displayed lower E-cadherin expression. (C) HCC tumors with lower miR-30a level showed higher Vimentin expression. Low-miR-30a ($n = 32$) and high-miR-30a expressing tumors ($n = 31$) were distinguished as described in Fig. 2. Expression of E-cadherin and vimentin was quantified in immunohistochemical staining using an IOD by Image-Pro Plus 6.0 software. For (B and C), the Mann Whitney test was performed. The central horizontal line indicates the mean value and the error bar indicates SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

Although altered miRNA expression has been found in various types of malignancies [22–24], the molecular mechanisms by which miRNAs modulate the behavior of cancer cells are still largely unknown. Here we demonstrated that downregulation of miR-30a was a frequent event in HCC tissues and could serve as an independent prognosis predictor for HCC patients. Furthermore, downregulation of miR-30a contributed to cancer cell migration and invasion, which was associated with EMT. Our findings also

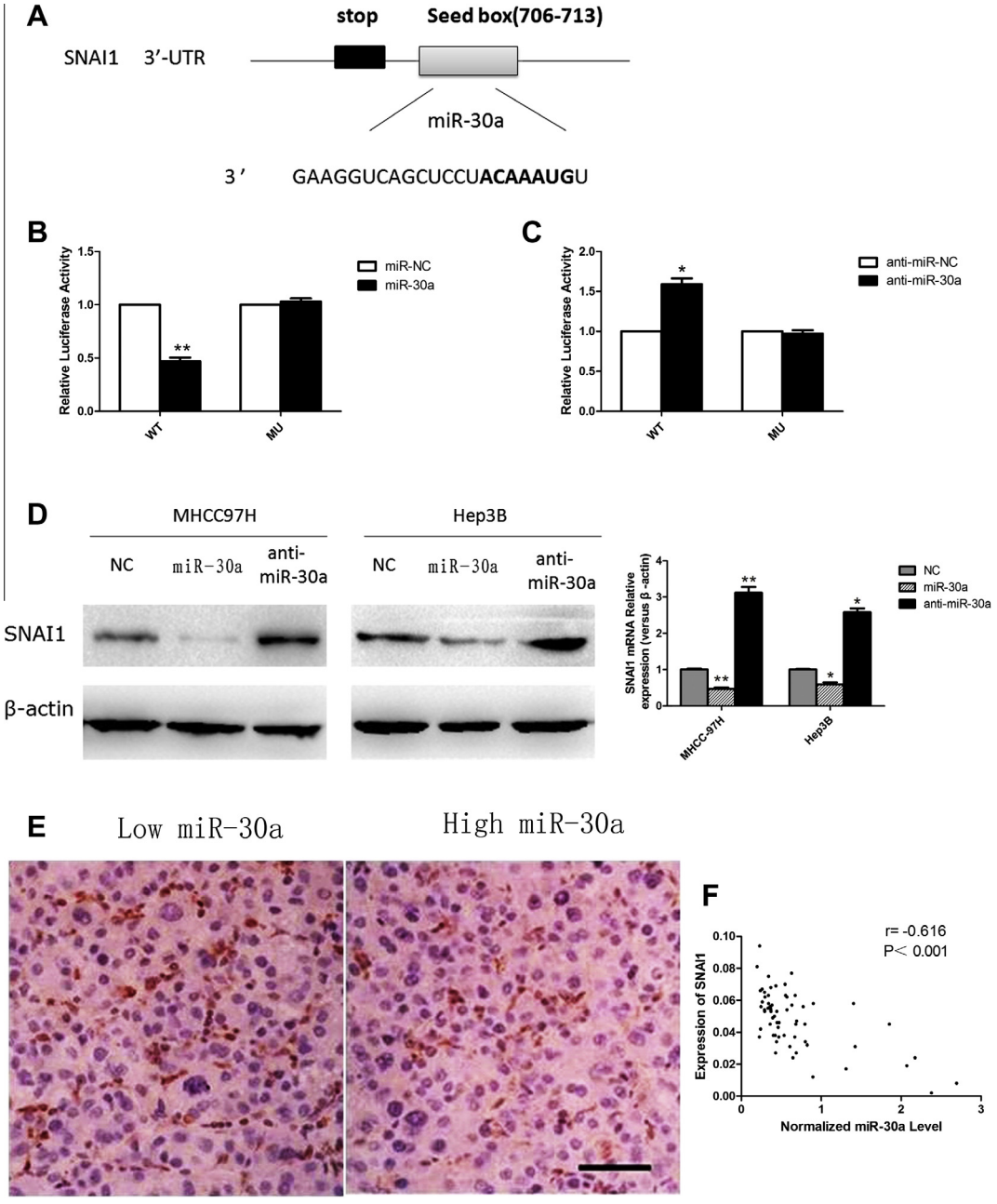

Fig. 6. SNAI1 is a direct target of miR-30a. (A) SNAI1 is predicted as a target of miR-30a. (B) Luciferase assays of cells cotransfected with miR-30a mimic or miR-NC (negative control), pRL-TK and firefly luciferase reporter plasmid containing the wild-type or mutant 3′-UTR of SNAI1. (C) Luciferase assays of cells transfected with anti-miR-30a or anti-miR-NC, pRL-TK and the firefly luciferase reporter comprising the wild-type or mutant 3′-UTR of SNAI1. pRL-TK expressing *Renilla* luciferase was cotransfected as an internal control, and the firefly luciferase activity of each sample was normalized to the *Renilla* luciferase activity. The normalized luciferase activity of miR-NC or anti-miR-NC transfectants was set as relative luciferase activity 1, therefore no error bar was shown for miR-NC and anti-miR-NC transfectants. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with miR-NC or anti-miR-NC transfectants. (D) Effects of miR-30a overexpression or suppression on endogenous SNAI1 levels. Forty-eight hours after transfection as indicated, MHCC97H and Hep3B cells were harvested and analyzed by Western blot and qRT-PCR. (E) Immunohistochemistry staining of SNAI1 in HCC tissues. Representative immunohistochemical staining in HCC tissues with low miR-30a and high miR-30a expression are shown. Scale bar = 50 μm. (F) Inverse correlation between miR-30a and SNAI1 levels in HCC tissues. Expression of SNAI1 analyzed by immunohistochemical staining was quantified using an IOD by Image-Pro plus software (Version, 6.0). Mature miR-30a levels were examined by qPCR analysis and normalized to RNU6B expression. Statistical analysis was performed using Pearson's correlation coefficient.

showed that ectopic expression of miR-30a in HCC cells could prevent EMT and inhibited cell migration and invasion. In addition, we further identified SNAI1 as a functional target of miR-30a and demonstrated an involvement of SNAI1 in the effects of reduced miR-30a on promoting cell migration, invasion and EMT changes. Our data suggest a fundamental role for miR-30a in EMT as well as migration and invasion of HCC cells, and implicate the potential application of miR-30a in prognosis prediction and cancer therapy.

To clarify the clinical prognostic significance of miR-30a, we examined the expression of miR-30a in 63 HCC samples to examine potential links between the miR-30a expression and survival prognosis, as well as cancer metastasis. Kaplan–Meier survival analysis showed that the level of miR-30a was correlated with DFS, with HCC cases expressing lower levels of miR-30a exhibiting shorter DFS. Downregulation of miR-30a was previously been reported to be a poor prognostic marker in multiple types of cancer [9,11], which is consistent with our results. Our conclusions indi-

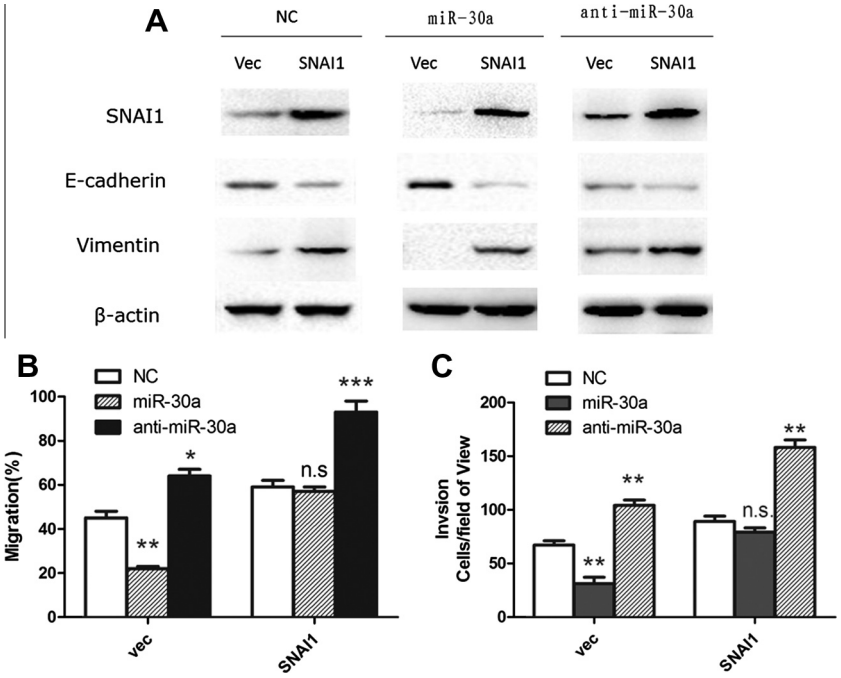

Fig. 7. MiR-30a exerts its function by suppressing SNAI1. (A) Ectopic expression of SNAI1 abrogates the effect of miR-30a on changes in protein markers associated with EMT, and further enhances the effect of anti-miR-30a on EMT changes in MHCC97H cells. After cotransfection of MHCC97H cells with the indicated RNA duplex for 3 days, protein markers associated with EMT were analyzed by Western blot. β-Actin was used as an internal control. (B and C) Upregulation of SNAI1 abrogates the inhibiting effect of miR-30a on migration and invasion, and strengthens the promoting effect of anti-miR-30a on migration and invasion. Three days after cotransfection with the indicated RNA duplex, the migration and invasion ability of MHCC97H cells were evaluated by wound healing assay and transwell invasive assay, respectively. Non-transfected cells were used as a negative control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, comparison between two groups as indicated.

cate that miR-30a level could be a predictive marker for clinical outcome in HCC. Prospective clinical studies are necessary to confirm whether miR-30a is a reliable clinical predictor of prognosis for cancer patients.

Migration, invasion and metastasis are major cellular processes that must be circumvented to prevent malignant tumor progression. Downregulation of miR-30a may enhance the migration, invasion and metastases of cancer cells, and in turn, stimulate the development of tumors. EMT is a key step that facilitates tumor migration and invasion. Increasing evidence has supported an important role for miRNAs in regulating EMT [25,26], which encouraged us to study the role of novel miRNAs in the regulation of EMT. In this study, we employed MHCC97H and Hep3B cells to examine how miR-30a exerts its function and modulates the migration and invasion of cancer cells, and our results provide new evidence for roles of miR-30a in cancer cell migration and invasion. Our findings show that ectopic expression of miR-30a in HCC cells could prevent EMT and inhibit cell migration and invasion, and downregulation of miR-30a can facilitate EMT change and promote migration and invasion in HCC cells. These results indicate that miR-30a has important roles in regulating EMT. Furthermore, our data in 63 paired HCC tissues showed that the expression of miR-30a was lower in patients with metastasis than patients without metastasis, revealing a clear correlation between miR-30a expression and HCC metastasis. Together with the in vitro findings showing that reduced miR-30a in HCC cell lines promotes migration and invasion, these results further confirmed the roles of miR-30a in migration, invasion and metastasis of HCC cells.

To completely understand the specific biological functions of miR-30a, identification of targets of miR-30a is critical. We used the miRNA target prediction program and a dual-luciferase report assay to demonstrate that miR-30a directly down regulates SNAI1 by binding its 3′-UTR. Furthermore, ectopic expression of miR-30a resulted in downregulated expression of endogenous SNAI1 protein and mRNA, while targeted knockdown of miR-30a increased the expression of SNAI1 protein and mRNA. In addition, we observed significant inverse correlations in clinical samples between miR-30a and SNAI1 expression levels in patient tumors, which supports the regulation of miR-30a on SNAI1 observed in vitro. Together these results indicate that SNAI1 is a direct physiological target of miR-30a.

SNAI1 is a well-known transcriptional repressor of E-cadherin that binds to three E-boxes in the human E-cadherin promoter [27]. The adhesion protein E-cadherin plays a central part in the process of epithelial morphogenesis, and loss of E-cadherin expression is a key event in EMT. Consistent with this, ectopic expression of miR-30a repressed EMT, accompanied by increased levels of E-cadherin. Furthermore, in the rescue experiment, overexpression of SNAI1 abrogated the inhibitory effect of miR-30a on EMT and strengthened the effect of anti-miR-30a on promoting EMT in MHCC97H cells. This study provides a new link between miR-30a and SNAI1 in the regulation of HCC cell EMT programs. EMT is a crucial step in tumor invasion and metastasis, therefore inhibition of EMT represents a very promising therapeutic strategy to prevent tumor invasion and metastasis [28]. In this study, we found that forced expression of miR-30a in HCC cell lines could decrease migration and invasion abilities. This finding might provide a valuable tool in the development of new therapeutic avenues against invasive and metastasis.

In the current study, we investigated the potential role of miR-30a in tumor progression and its underlying mechanisms. Our results suggest that downregulation of miR-30a may play an essential role in the development of HCC, and that miR-30a may be employed as a prognosis marker and therapeutic target for HCC. Nevertheless, these data should be further validated in independent cohorts and prospective trials.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.037.

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